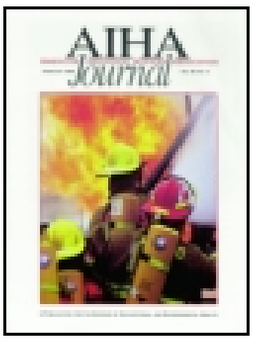

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Preparation and Examination of Proposed Consensus Reference Standards for Fiber-Counting

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This study provided standard reference materials for fiber-counting by phase-contrast microscopy (PCM). PCM is subject to many sources of variation, including those dependent on the microscopist, so reference standards cannot be produced that are traceable to national or international standard units. Consensus standards using a "true value" agreed on by a number of laboratories may be acceptable. Reference slides for fiber-counting can be prepared using a proprietary process of grid overlay in which the fields of view defined by the grids are identifiable and relocatable. Multiple microscopists then can examine exactly the same areas of samples, reducing one source of potential variation. Twelve slides prepared from proficiency test samples of the American Industrial Hygiene Association (AIHA) Industrial Hygiene Laboratory Quality Program were used in this study, four each of chrysotile asbestos, amosite asbestos, and man-made mineral fibers. Five microscopists from AIHA-accredited laboratories, plus the inventor of the grid process, examined the slides in a blind study. This group represented commercial analytical companies, in-house corporate laboratories, research institutions, and universities. The six microscopists met to obtain consensus agreement on the fibers in each designated field classified as countable under National Institute for Occupational Safety and Health Method 7400. Slides and documentation were forwarded to AIHA for training or other purposes. Examination of the results by statistical methods showed that some microscopists' results were significantly different from others, even though all analysts would have been considered proficient with respect to the final consensus values. Although the reasons for the outliers are complex, this procedure may have value in selecting reference laboratories in proficiency test schemes, possibly leading to more defensible "true" values and tighter limits of variation.

Keywords: asbestos, fiber-counting, phase-contrast microscopy, quality assurance, reference material, relocatable field

A current theory suggests that the fiber dose, fiber dimensions, and fiber durability in lung fluid are the three primary factors affecting fiber toxicity.[1] Fiber length appears to be associated with the potential for fatal macrophage ingestion and fiber diameter with the probability of deposition in specific areas of the lung. The asbestiform mineral habit is characterized by fibrils (about 0.03 μm diameter) packed together. Fibers from asbestiform minerals may be isolated or bundles of fibrils. Asbestiform minerals, as well as some other materials with a similarly fibrous habit, are considered potentially hazardous to health, and it is necessary to characterize personal exposures to these fibers. Sampling of particles with diameters greater than about 1 μm with impingers, followed by counting all particles, was the traditional method of dust sampling. However, its application to asbestos fiber exposure was questionable because there was poor correlation between particle counts and health effects.[2] The method was replaced by cellulose membrane filter collection, in which fibers on the filter can be

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TABLE I. Intercomparison of Microscopists (A–F) for Amosite

Microscopist	A	B	C	D	E	F
A						
B	0.5091					
C	0.0786	0.2713				
D	0.8867	0.4222	0.0574			
E	<.0001	<.0001	<.0001	<.0001		
F	0.0114	0.0608	0.4374	0.0075	<.0001	

Note: Numbers are probabilities; the lower the number the more probable the difference between analysts is not caused by chance.

identified through phase-contrast optical microscopy (PCM).[3] The filters are rendered transparent (''cleared'') by means of acetone, in which case they are fixed with triacetin,[4] or by dimethylformamide, followed by fixing in Euparal resin.[5] Triacetin slides may last for several years, but Euparal slides appear to have an even longer shelf-life.[6] PCM at a nominal 400× magnification allows the detection of fibers at least 0.25 μm diameter, depending on the fiber type.[2] A test slide has been developed to allow a check of proper alignment and magnification in the microscope,[7] together with an eyepiece graticule to standardize the counting field as a circle of 100 μm diameter,[8] and rules have been formulated for the identification and enumeration of fibers.[3] The method does not detect all fibers because of the limitations of microscopy, so that the method is only an index of exposure.[2] Larger numbers of fibers, especially chrysotile, may be observed at higher magnifications or with transmission electron microscopy (TEM).[9]

There are many issues involved in the optical detection and recognition of fibers, including:

(1) the set-up of the microscope;

(2) the quality of the contrast between the fibers and the background, especially with thin chrysotile fibers, causing some fibers to be missed;

(3) the visual acuity of the microscopist and the care with which the field is traversed;

(4) the fact that fibers may lie in different focal planes, requiring constant adjustment of the focus to ensure no fibers are missed;

(5) the ability of the microscopist to accurately size fibers, causing fibers close to the 5 μm minimum length limit (especially for amosite) or 3 μm width limit (for man-made mineral fibers [MMMF]), or those close to the 3:1 minimum aspect ratio, to be either over- or undercounted; and

(6) the ability of the microscopist to properly apply the counting rules to, for example, split fibers, bundles, and fibers crossing boundaries.

A method that enables these errors to be assessed would be very useful, both for research and training purposes. Such a method now exists and is described here.

QUALITY ASSURANCE

Most methods require individual microscopists to recount about 10% of field samples and to set some samples aside for routine recounts over time to ensure consistent counting procedures. Unfortunately, a microscopist is unlikely to come back to exactly the same fields of view as in the original counts, resulting in an uncontrollable component of the variation. A mechanism to allow recounts of the same areas would remove this additional component and allow a better assessment of the true variation of the microscopist. Interlaboratory sample exchange programs have been shown to be more important in ensuring agreement between laboratories than similarity in details of counting rules.[10] Again, the lack of relocatable fields of the view imparts an additional component to the variation in the results.

In the past few years a technique has been under development for improving the accuracy of fiber-counting by allowing exactly the same fields of view to be examined by multiple microscopists or the same microscopist on different occasions.[6,9,11] The method involves the deposition of an almost transparent TEM grid onto the cleared sample. Included with the grid are coordinates allowing each grid square to be relocatable. Photomicrographs of typical grid openings superimposed on chrysotile and amosite samples have been published.[11] Slides prepared in this manner have been used in a Canadian proficiency test program for many years. The main errors affecting the counts of each fiber type (chrysotile, amosite, and MMMF) have been evaluated through the examination of large numbers of slides by large numbers of participants in this program. A scoring system for identifying the performance of microscopists has been developed[12] based on errors compared with a reference value defined for each slide by the laboratory in which they were produced. This scoring system is being prepared for publication. When news of this technique reached the Proficiency Analytical Testing (PAT) subcommittee of the American Industrial Hygiene Association's (AIHA's) Industrial Hygiene Laboratory Accreditation Committee (IHLAC), their value for training purposes was quickly recognized. A proposal was accepted

TABLE II. Intercomparison of Microscopists (A–F) for Chrysotile

Microscopist	A	B	C	D	E	F
A						
B	0.1142					
C	0.0469	0.6823				
D	0.0669	0.7994	0.8766			
E	0.0187	<.0001	<.0001	<.0001		
F	<.0001	0.0029	0.0100	0.0063	<.0001	

Note: Numbers are probabilities; the lower the number the more probable the difference between analysts is not caused by chance.

TABLE III. Intercomparison of Microscopists for MMMF

Microscopist	A	B	C	D	E	F
A						
B	0.1486					
C	<.0001	0.0063				
D	0.5074	0.0352	<.0001			
E	0.5865	0.0469	<.0001	0.9053		
F	0.1102	0.8784	0.0069	0.0239	0.0323	

Note: Numbers are probabilities; the lower the number the more probable the difference between analysts is not caused by chance.

to obtain some slides and to have them examined by a number of microscopists from AIHA-accredited laboratories and to have the microscopists come together to agree on the fibers defined in accordance with the National Institute for Occupational Safety and Health (NIOSH) Method 7400, and then to have these slides, together with the documentation available through AIHA for training or other purposes.[13] Once examined in this way the slides are candidates for consensus reference standards.

Proficiency test samples are sent out to laboratories in both national and international testing schemes.[14,15] It is not possible to make these samples exactly identical. Part of the variation is due to the production of the samples, and the aim of the test providers is to render this variation as small as possible. A reference mean is then developed for the sample set, together with an acceptable variation about that mean with which the test participant's results can be compared. There is a major issue regarding both the determination of the "true" value, and the setting of the acceptable limits around that value, which has been discussed at length in the literature.[16,17] This discussion has resulted in one provider, AIHA, changing its methods for determining the "true" value and limits several times in the recent past. Currently, AIHA uses a group of "reference laboratories" to derive the mean and the limits, but the selection criteria for the "reference laboratories" is possibly not fully defensible. A more robust mechanism for determining a reference laboratory would be very useful. During the preparation of the consensus reference slides, it became apparent that statistical analysis of the intragroup precision could identify those members of the group that were outliers compared with the others. This leads to the possibility that consensus reference slides can be used to identify those participants of the proficiency test program that are most suitable to be included in the reference population (i.e., those who are not determined to be outliers after consensus reference slides are examined).

METHODS AND MATERIALS

Twelve slides were manufactured from IHLAC PAT samples in accordance with the proprietary process that imprints a coded TEM grid onto the samples as described previously. The slides were prepared in Euparal mounting medium,[18] and it is believed that they will last for many years. Four of the slides contain chrysotile asbestos, four contain amosite asbestos, and four contain MMMF.

After manufacture, the inventor of the process, Thomas W.S. Pang of Ryerson University, Ontario, Canada, examines the slides and provides "certified counts" of the fibers present in specific, identifiable fields on the slides. These counts accompany the purchase of the slides. The number of fields was selected so as to give approximately 100 fibers per slide. The slides, together with the certified count data, were obtained from Omega Specialty Instruments, Waltham, Mass. Pang's certified count data were used as the results from one microscopist in the study but were not given any more weight than that in the final analysis.

Five other microscopists from AIHA-accredited laboratories also examined the same areas of the slides as Pang and recorded their own fiber counts in a blind study. The microscopists were Bruce Harvey of Research Triangle Institute, N.C.; Cecile Fook of Merck Corp. in New Jersey; Julie McCabe of IRSST, Quebec, Canada; Kuntal Parikh of Clayton Corp. in Georgia; and Peter Steen of Datachem Corp. in Utah. These five, together with Pang and an independent referee (Harper), convened a resolution meeting at the IRSST. During the 2-day meeting, agreement was reached as to the visible fibers definable according to the applicable counting rules in NIOSH Method 7400. The certified counts documentation provided by Pang has been updated to reflect this consensus and is included with the slides, which are currently housed at AIHA headquarters. It should be noted that Pang's certified count results were not outliers.

Three questions were asked of the statistical analysis:

(1) Within each fiber type, is it possible to define microscopists as outliers from the group?

(2) Within each fiber type, is it possible to define microscopists as outliers from the consensus reference standard?

(3) Within each fiber type, what is the variance both with and without the outlier microscopists identified in questions 1 and 2?

Question 1 was answered using analysis of variance, and question two was answered using Dunnett's test, both from SPSS version 10. The variance in question three was expressed as the standard deviation of the total number of counts from the six analysts.

TABLE IV. Amosite Variation with Respect to Consensus Value with and Without Outlier Analysts

Slide No.	RSD, All Analysts[A]	RSD, All Minus E[A]
108-1A	24.5	11.1
122-2B	21.3	13.1
122-4C	18.0	10.7
122-4F	22.8	8.73

[A]RSD = % relative standard deviation.

TABLE V. Chrysotile Variation with Respect to Consensus Value with and Without Outlier Analysts

Slide No.	RSD, All Analysts[A]	RSD, All Minus E, F[A]
107-3B	19.5	8.14
115-2B	24.6	8.02
119-1C	9.96	5.77
119-2C	20.4	19.7

[A]RSD = % relative standard deviation.

TABLE VI. MMMF Variation with Respect to Consensus Value with and Without Outlier Analysts

Slide No.	RSD, All Analysts[A]	RSD, All Minus C[A]
123-3A	13.0	6.80
123-4A	16.9	14.5
123-4B	13.6	8.79
123-4C	13.0	13.3

[A]RSD = % relative standard deviation.

RESULTS AND DISCUSSION

It should be kept in mind, whatever the use of these slides, that they are not identical to proficiency test samples because of the grid overlay, which causes the field of view to be slightly darker than normal. However, the examining team did not feel this was a serious drawback to fiber recognition (and future samples will have the grid reversed, so that the fields of view will be clear). The fibers counted in this study were those that fell completely or partially within the grid fields. The area of each field is larger (0.01025 mm^2) than that of the Walton-Beckett graticule area (0.00785 mm^2). The boundary of the grids is not as sharp as that of the graticule. Thus, the following convention was agreed on in determining whether a fiber should be counted. If a fiber had one end wholly outside the field and one end in the diffuse boundary area, it was considered out of the field. If a fiber had one end wholly within the field and one end in the diffuse boundary area, it was considered in the field. Only those fibers with one end completely outside and one end completely inside (i.e., no ends in the diffuse boundary area) were considered to be crossing the boundary. This is a slightly different definition from that used by Pang in his initial review of the slides, causing a slight difference in comparing his certified count with that of the group, but causing an average "error" rate of less than 5% for the majority of the slides, and often with almost equal numbers of positive and negative "errors," so that the total counts did not change significantly.

As discussed earlier, important factors in defining a fiber are the length and width dimensions and ratios. Amosite fibers frequently (and chrysotile fibers sometimes) are close to 5 μm in length. NIOSH 7400 defines fibers as being greater than (and not equal to) this value. MMMF frequently are close to 3 μm in width. NIOSH 7400 defines these fibers as being less than (and not equal to) this value. Care must be taken in the measurement of fibers within 0.1 μm of these values. Although the group took much care and effort in such measurements, they are visual comparisons, and therefore there is no warranty that all measurements are free from error.

The other controversial issue during the resolution meeting was the identification of scratches or similar defects on the slides. The morphology of some "fibers" resembled scratches, and those images were further examined by altering the focus of the microscope, but an unequivocal agreement that they were defects was required before they were counted as such. The default approach was to include them in the fiber count. This was a significant issue for only 1 of the 12 slides.

Finally, it should be emphasized that very fine fibers of chrysotile are easily missed without very careful inspection. The group agreed that this is probably the most common error in the undercounting of fibers. Thus, for chrysotile the final consensus results included more fibers than expected than from the mean of the six microscopists.

Statistical analysis of the group intercomparison (Tables I–III) gave essentially the same results as the comparison of the group to the consensus results, indicating that to identify outlier laboratories in future studies it may not be necessary to derive a consensus value. For amosite one laboratory (E) gave significantly higher results than most others. The reason for this was identified principally as the overcounting of fibers at or below the 5 μm length limit. The same microscopist also gave significantly higher counts for chrysotile, although in this case the cause was a combination of overcounting short fibers and also identifying very thin fibers that had escaped the view of the other microscopists. Many of these thin fibers were validated in the resolution meeting. For chrysotile a further microscopist (F) was identified as giving results that were lower than the consensus mean. Although not significant on Dunnett's test, removal of this analyst did cause the variation within the other four microscopists to become insignificant. This analyst apparently missed more fine fibers than the others. For MMMF yet another microscopist (C) was found to be significantly higher than the others, and the reason in this case was the inclusion of some fibers at or just over the 3 μm width limit. It must be stressed that none of these microscopists would have been considered nonproficient in a proficiency test program based on these results, and that these tests are examining variation among a much smaller and tighter pool of microscopist results than is normally encountered in AIHA's PAT program. However, the power to discriminate between the microscopists is apparent. The overall variations of total fiber counts both with and without the outlier microscopists are provided in Tables IV through VI. For each fiber type, the pooled relative standard deviations without the outliers (amosite 11.0%, chrysotile 11.8%, MMMF 11.3%) fall within the 12.8% (95% confidence limit) required for an unbiased air sampling method, as preferred by NIOSH. This is a further indication of possible uses for this technique in the future.

CONCLUSIONS

Six microscopists examined the fibers found within specified relocatable grid areas on 12 proficiency test slides representing three fiber types (amosite, chrysotile, and MMMF). In the blind portion of the study, at least four microscopists agreed without significant differences on the number of fibers present on each of the 4 slides in each fiber type. Different microscopists were identified as outliers, depending on the fiber type, and the reason for the outliers. The causes of outlier counts were as noted in prior studies using this technique. All six microscopists agreed on a consensus reference count for each field on each slide (approximately 400 fields in total). When each microscopist's individual result was compared with the consensus reference standard, the outlier results did not change significantly. Removal of these outliers gave pooled standard deviations for the counts within the limit specified for an unbiased air sampling method. The slides and their final consensus documentation should provide a useful tool for the training of microscopists in the identification of countable fibers. They also may have a role in helping to define those laboratories that should be used as the reference for determining the "true" value for proficiency test samples. The appropriate AIHA technical committees undoubtedly will suggest and direct their future applications.

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